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Importance of the chemical defenses and sugars in the feeding preference of *Paracentrotus lividus* over two sympatric template seagrass species

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ABSTRACT

Herbivory is a fundamental process regulating the functioning of the ecosystems both in land and marine systems. Few decades ago, herbivory was thought to play only a minor role in seagrass dominated areas, while currently its importance has risen. However, the complex interrelationships between seagrasses and their consumers are not yet fully understood. For instance, seagrasses can tune up morphological, biomechanical, nutritional, and chemical traits in order to reduce the palatability of their leaves and therefore curtail tissue losses, but the final tradeoff of such changes may depend on herbivore guilds. This work focuses on the relative importance of nutritional *versus* chemical traits in the feeding behavior of the sea urchin *Paracentrotus lividus*, a temperate generalist mesograzer, over two sympatric seagrass species (*Cymodocea nodosa* and *Zostera noltei*). To do that, a set of no-choice and multiple-choice feeding assays were conducted using freeze-dried plants agar-based diets (i.e. whole nutritional properties), polar extracts of both species (i.e. soluble sugars and phenolic natural products), purified phenolic natural products, and also three different concentrations of soluble sugars. Later, a chemical identification and quantification of the phenolic natural products present in the extracts were performed to assess their ecological role as deterrents. Results clearly indicated that the feeding behavior of this generalist herbivore, once overlooked the structural, morphological and biomechanical traits, is mainly determined by nutritional properties, while the presence of phenolic compounds has only a minimal effect on its feeding behavior. In addition, although this study showed that sugars had a positive effect over *P. lividus* consumption rates, we demonstrated for the first time the deterrent properties of rosmarinic acid and the sulphated flavonoids produced by *Z. noltei*, which were able to reduce the attractive effect of sugars in the feeding preference of this generalist herbivore.

1. Introduction

Plant–herbivore interactions are fundamental to regulate ecosystem functioning, population dynamics, and community organization (Marone and Croone, 2006). Herbivores are involved in the transference of matter and energy from primary producers to higher trophic levels, affecting both the physical structure of the community and ecosystem, as well as the productivity of the habitat (Poore et al., 2012; Chapin et al., 2011).

Within coastal vegetated habitats, seagrasses are the basis of one of the most species-rich and relevant ecosystems (Short et al., 2011). Seagrasses are rooted, flowering plants adapted to marine environments, that grow both at inter and subtidal soft sediment areas along the coasts of most continents (Short et al., 2007). Differing from the well-studied plant-herbivore interactions in terrestrial plants and seaweeds (Duffy and Hay, 1990; Coley and Barone, 1996; Paul et al., 2001; Toth and Pavia, 2007; Johnson, 2011; Fürstenberg-Hägg et al., 2013; Gong and Zhang, 2014), data about seagrass-consumer interactions are much scarce. In this regard, herbivory was firstly proposed to have only minimal impact on these marine plants due to the alleged unpalatability of their tissues (Thayer et al., 1984), and the decrease in the populations of major seagrass consumers such as waterfowl, sirenians, fishes and sea turtles (Jackson et al., 2001; Valentine and Duffy, 2006). However, later studies showed that herbivory had been greatly underestimated and that direct grazing on seagrasses, although context dependent, may be substantial (Valentine and Duffy, 2006).

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It is generally accepted that all plants, both terrestrial and aquatic, have developed diverse strategies to modify their palatability and thus reduce their vulnerability to herbivore attacks (Agrawal and Fishein, 2006; Agrawal, 2011; Carmona et al., 2011). In particular, structural, nutritional, and chemical traits are known to play a crucial role in the palatability of seagrasses and, therefore, in their consumption (Valentine and Heck, 2001; Heck and Valentine, 2006; Vergés et al., 2007a, 2018; De los Santos et al., 2012; Martínez-Crego et al., 2016), although the relative importance of these traits varies depending on the herbivore guilds and latitude (Lefebvre et al., 2000; Moran and Bjorndal, 2007; Vergés et al., 2007a, 2011, 2018; Prado and Heck, 2011). Structural defenses in seagrasses encompass characteristics such as changes in blade morphology (i.e. width or thickness) or toughness (i.e. fibers, lignin, etc), with many herbivores discriminating among seagrasses based on such traits (Vergés et al., 2007b; Prado and Heck, 2011). However, for some generalist herbivores, including sea urchins, fishes, mammals or birds, the structural traits may be not so relevant and feeding decision relays on nutritional traits and chemical defenses presence (Buchsbaum and Valiela, 1987; Paul et al., 1990; Meyer et al., 1994; Preen, 1995; Heck and Valentine, 2006; Prado and Heck, 2011; Vergés et al., 2007a, 2011; Tomas et al., 2011; Steele and Valentine, 2012; Sieg and Kubanek, 2013). The relative importance of nutritional *versus* chemical traits in seagrasses uses to be examined by feeding choice assays, where different food sources are offered to herbivores (e.g. seagrass species), either fresh or as freeze-dried material included in an agar matrix (Paul et al., 1990; Goecker et al., 2005; Prado and Heck, 2011; Tomas et al., 2011; Vergés et al., 2011; Steele and Valentine, 2012; Jiménez-Ramos et al., 2018b). Afterwards, the feeding choice of the herbivores is related *ad hoc* with some of the nutritional traits measured (e.g. nitrogen, carbon, protein, carbohydrate, lipid, caloric and/or organic matter contents) and/or with the total content of chemical compounds. The relative contribution of nutritional and chemical traits using such approach cannot be certainly isolated, since plants bear a specific combination of both traits that usually co-varies (Buchsbaum et al., 1990; Goecker et al., 2005; Tomas et al., 2011; Vergés et al., 2018). A way to unravel such inconvenience has been to include in the feeding assays the crude extracts obtained from seagrass leaves, mainly by using polar extraction solvents such as methanol, and mixtures of water with methanol or ethanol (Harrison, 1982; Meyer et al., 1994; Vergés et al., 2007a, b) and, in some instances, using less polar mixtures of organic solvents (Paul et al., 1990; Vergés et al., 2007a). Overall, these seagrass extracts are assumed to be devoid of most of the nutritional constituents (nitrogen rich compounds, proteins, carbohydrates, etc) and contain the chemical compounds known as secondary metabolites. In the assays, the chemical compounds recovered in the crude extracts are frequently described as phenolic compounds (e.g. Vergés et al., 2007a; b, 2018), and the bulk concentration is considered as a direct measurement of the deterrent activity (see reviews by Sieg and Kubanek, 2013; Zidorn, 2016). However, this procedure has several confounding weakness that need to be examined in order to make a proper interpretation of the results. Extractions using mixtures of water-acetone or water-ethanol or even pure methanol and ethanol are commonly used to recover polar compounds with biological functions, including phenolic acids, flavonoids, or even some tannins (Stalikas, 2007; Dai and Mumper, 2010). However, also a fraction of the soluble sugars (mono- and disaccharides) may be captured in these polar extracts, while pigments (chlorophylls and carotenoids), waxes, sterols, and many lipids can be retained in the non-polar extracts (Stalikas, 2007). Therefore, when using such polar crude extracts in the feeding assays, at least some sugars may be present in the diets. Since these non-structural carbohydrates can be also an important nutritional trait (Buchsbaum and Valiela, 1987; Prado and Heck, 2011) that could induce an active feeding behavior in the herbivores, overlooking its presence in the extracts-based diets may introduce an unexpected confounding factor in the interpretation of the results (i.e. the effects caused by the chemical defenses). Moreover, most studies often rely on the measurement of total phenolic concentrations to assess chemical defenses in seagrasses, with the deterrent function being only investigated at the level of compound classes (e.g. phenolic compounds, flavonoids, tannins) (Goecker et al., 2005; Vergés et al., 2007b, 2018, 2018; Tomas et al., 2011) and not at the level of chemically defined metabolites. However, minor differences in the molecular structure of phenolic compounds can lead to dramatic changes in their ecological function (Appel, 1993; Salminen and Karonen, 2011; Sieg and Kubanek, 2013; Zidorn, 2016), and therefore the total phenolic concentration may overestimate how well defended is a seagrass, since most of the phenolic metabolites of the plant do not play a role in the defense against herbivores (Sieg and Kubanek, 2013; Zidorn, 2016; Papazian et al., 2019). Thus, as suggested by Sieg and Kubanek (2013) in their recent review, the identification of individual compounds and subsequent determination of their ecological role will allow researchers to accurately measure plant investment in chemical defenses against a variety of herbivores, pathogens, and other threats.

In multispecific populations where some species share structural, morphological, and biomechanical traits (Mariani and Alcoverro, 1999; Hemminga and Duarte, 2000; Ballorain et al., 2010; Kelkar et al., 2013; Hernández and Tussenbroek, 2014; De los Santos et al., 2016), or even in monospecific populations where spatial explicit differences in biotic and abiotic factors can be found (Brun et al., 2003a; Prado et al., 2010; De los Santos et al., 2013; Gera et al., 2013), the active feeding decision of herbivores, as aforementioned, may be determined by the combination of nutritional traits, phenolic compounds presence, and sugars content in seagrass tissues. All these factors are known to vary seasonally (Pirc, 1989; Pérez and Romero, 1994; Brun et al., 2003a; Steele and Valentine, 2012; De los Santos et al., 2013; Enerstvedt et al., 2017; Manck et al., 2017), and also in response to anthropogenic stressors like nutrient enrichment (Brun et al., 2002; La Nafie et al., 2012; Martínez-Crego et al., 2016; Jiménez-Ramos et al., 2017), light reduction (Brun et al., 2003b; La Nafie et al., 2013; Silva et al., 2013), temperature increase (Vergeer et al., 1995; Jiménez-Ramos et al., 2017; Egea et al., 2018), acidification (Martínez-Crego et al., 2014; Jiménez-Ramos et al., 2017; De los Santos et al., 2019; Arnold et al., 2012) or salinity change (McKone and Tanner, 2009; Villazán et al., 2015; Jakobsson-Thor et al., 2018) among others. Therefore, the feeding preference in nature over seagrass communities may be altered at both large and small scales depending on the extent at which the stressor is acting and altering not only structural, morphological, and biomechanical traits, but also nutritional properties, sugar and natural product concentrations.

This study aims to explore the relative importance of nutritional and chemical traits in the feeding behavior of a generalist herbivore on seagrasses. For this purpose, we used as model the sea urchin *Paracentrotus lividus,* and the two template sympatric seagrass species *Cymodocea nodosa* and *Zostera noltei,* which are known to be a food resource for herbivores and play an important role in the food web (Cebrián et al., 1996; Fernández et al., 2012; Martínez-Crego et al., 2016). In addition, the importance of sugars content in the feeding decision of this generalist herbivore was experimentally tested for the first time, and deterrent properties of phenolic compounds in both species were investigated at the chemical compound level. To do that, a set of no-choice and multiple-choice feeding assays were conducted using freeze-dried plants agar-based diets and polar extracts of both species. Moreover, the presence of soluble sugars in both extracts was analyzed, and their effects on the feeding behavior of *P. lividus* experimentally tested. A posterior chemical identification and quantification of the phenolic compounds present in the extracts is performed, including the analysis of their potential ecological role as deterrents.

2. Materials & methods

2.1. Collection of sea urchins

Sea urchins (*Paracentrotus lividus*) were collected from a nearby

rocky shore, La Caleta, in Cádiz (SW Spain, 36°31′39″N; 6°18′46″W), where a stable population of *P. lividus* inhabits. Once permission was granted by local environmental authorities, 115 individuals were collected at a depth of 2 m. Harvesting was carefully carried out by snorkeling, avoiding damage to the organisms. Sizes varied between 3 and 5 cm in diameter (adult size). Collected organisms were kept in cooled containers with seawater and transported to the laboratory, where they were placed in aerated tanks at a temperature of 18 °C. Sea urchins were fed with *Ulva* sp. for 4 days until the beginning of the experiment to allow their acclimation to laboratory conditions. During this time, the photoperiod was set at 8:16h (light:darkness) because *P. lividus* usually exhibits nocturnal activity (Boudouresque and Verlaque, 2001).

2.2. Collection of plant material

Cymodocea nodosa (Ascherson) and *Zostera noltei* (Hornemann) plants were collected at the inner area of the Bay of Cadiz (SW Spain; 36°28′10.41″N, 6°14′57.60″O) on April. Both seagrasses inhabit the shallow area, at 0.4 and −0.5m above and below the chart datum (lowest astronomical tide) (Vergara et al., 2012). In order to obtain a representative sample of these plants in the natural environment, more than 200 intact vertical shoots from each species were randomly collected from an area of approximately 20 ha, trying to leave a minimum gap of 10 m between collected shoots. Therefore, our sampled shoots include the environmental heterogeneity in the area (i.e. depth, sediment properties, hydrodynamic forces, etc). Shoots were transported to the laboratory at the University of Cadiz, within the next 2h after collection in an ice chest. Upon arrival, leaves of each seagrass species were carefully cleaned with seawater and epiphytes were removed with soft laboratory paper to avoid damaging leaf surfaces. Leaves were subsequently separated from the rhizomes, pooled and finally separated in three replicates. Then, each replicate of fresh leaves from each species was divided into two portions, one (30 g) for freeze-drying and another (110 g) for chemical analyses.

2.3. Preparation of extracts from the leaves of Cymodocea nodosa and Zostera noltei for the feeding assays

Each replicate of fresh leaves (75 g fresh weight) was extracted with methanol (MeOH, 400 mL) under maceration with shaking and sonication for 5 min. The solution was filtered over paper and the residual plant material was subjected to two more extraction cycles following the same procedure. The resulting methanolic solution was evaporated under reduced pressure in a rotary evaporator to obtain an oily dark green extract. Subsequently, this extract was suspended in water (125 mL) and successively extracted with diethyl ether (Et_2O, 100 mL x 3 cycles), and then with *n*-butanol (BuOH, 50 mL x 3 cycles). The diethyl ether phases, which contained non-polar compounds, lipids, and pigments, were discarded. The *n*-butanol phases, which contained phenolic compounds and a fraction of the sugars of the plant, were combined and the solvent was evaporated under reduced pressure in a rotary evaporator to yield a yellowish solid extract. Each extract was analyzed by proton nuclear magnetic resonance (^{1}H-NMR, Agilent 500 spectrometer) using CD_3OD as solvent (chemical shifts in ppm, referenced using the solvent signal at δ_H 3.30).

2.4. Isolation of phenolic natural products for the feeding assays

The analysis of the extracts of both seagrass species by NMR indicated that only the extract of *Z. noltei* contained enough amounts of phenolic compounds to be isolated and tested in the feeding assays. Therefore, only the extract of *Z. noltei* was separated to obtain phenolic natural products by subjecting a portion of the extract (670 mg) to sephadex LH-20 column chromatography (29.0 × 2.5 cm), using methanol as eluent. The aliquots obtained were analyzed by thin-layer chromatography and ^{1}H-NMR. Those containing phenolic compounds were combined and the solvent evaporated in a rotary evaporator. The compounds obtained were used to prepare diets for the feeding assays.

In order to determine the amount of phenolic compounds required to be included in the diets, the natural concentration of these metabolites in fresh leaves of. *Z. noltei* was determined by quantitative analysis of the extracts by using ultra performance liquid chromatography-mass spectrometry (UPLC-MS). Fresh leaves of *Z. noltei* (*ca*. 3.0 g, three replicates) were extracted with methanol (30 mL x 3) and sonication (5 min). The solutions were filtered over paper, combined, and subsequently evaporated in a rotary evaporator to yield an extract that before analysis was cleaned on SPE-RP18 cartridges using methanol/water (9:1, v/v) as eluent. The solution obtained was evaporated in a rotary evaporator and dissolved in water at 50 μg/mL for injection in the UPLC-MS. Analyses were performed on an ACQUITY Ultra Performance LC system coupled to a Synapt G2 Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) source using the chromatographic and mass spectrometry conditions previously described in Manck et al. (2017). The single components were characterized by the retention time and the accurate molecular masses (Manck et al., 2017). For the quantification two calibration curves were constructed, one with commercial rosmarinic acid and another with pure apigenin-7-sulfate isolated from the extract (for the quantification of flavonoids).

2.5. Quantitative analysis of sucrose in the butanolic extracts

The content of sucrose in the butanolic extracts of *C. nodosa* and *Z. noltei* was determined by quantitative ^{1}H-NMR, using 1,3,5-trimethoxybenzene (Sigma-Aldrich TraceCERT) as internal standard (IS) and CD_3OD as solvent. The anomeric proton signal of sucrose (δ_H 5.38, 1H) and the aromatic protons signal of the IS (δ_H 6.07, 3H) were used for quantification.

2.6. Experimental set-up for feeding assays

2.6.1. Diets based on leaves, extracts, and phenolic natural products

Feeding assays were performed using agar-based artificial diets to examine whether the feeding pattern of a generalist herbivore (i.e. sea urchin) is driven by nutritional or chemical differences in plant tissues. Two diets were prepared with the freeze-dried leaves of *C. nodosa* and *Z. noltei* (LC and LZ, respectively), which retained all the nutritional traits, sugars, and phenolic natural products from each plant. Another two diets were prepared with the butanolic extracts of *C. nodosa* and *Z. noltei* (BEC and BEZ, respectively). These diets mainly contained sugars and phenolic natural products from each plant. Finally, a diet was prepared with the pure phenolic natural products isolated from the extract of *Z. noltei* leaves (diet PNP), since no phenolic compounds could be isolated from the extract of *C. nodosa* leaves (see subheading 2.4 and Results section). Following the method indicated by Goecker et al. (2005) the two first agar-based diets (LC and LZ) were prepared using 1.4 g of freeze-dried leaves (resulting of freeze-drying 6 g of fresh seagrass leaves), which were ground to a fine powder and then embedded in agar by combining with a solution of 135 mg of agar in 6.7 mL of distilled water at 80 °C. The mixture was poured into small moulds, and allowed to cool for 1 h in a refrigerator yielding an agar block of approximately 6 g for each diet. The same procedure was followed to prepare the other agar diets but replacing the freeze-dried seagrass leaves by the butanolic extract of *C. nodosa* (BEC), the butanolic extract of *Z. noltei* (BEZ) or the phenolic natural products (PNP). The amount of extract used to prepare BEC and BEZ diets was the corresponding to 6 g of fresh leaves (which was the amount of fresh leaves used in the two first diets, LC and LZ). Therefore, upon comparison with the LC and LZ diets, the BEC and BEZ diets should contain similar concentrations of phenolic natural products and a portion of the sugars, while nitrogen rich compounds, salts and other products were removed. The amount of phenolic compounds in the PNP diet was also the corresponding to 6 g of

fresh leaves of *Z. noltei*. Thus, the PNP diet contained a concentration of phenolic compounds equivalent to the LZ diet.

Therefore, five different agar-based artificial diets were prepared (Fig. 1): 1) freeze-dried *C. nodosa* leaves (LC), 2) freeze-dried *Z. noltei* leaves (LZ), 3) butanolic extract of *C. nodosa* (BEC), 4) butanolic extract of *Z. noltei* (BEZ) and 5) phenolic natural products (PNP). Additionally, a control diet of agar (AC) was prepared only with water and agar (2% weight:volume) to evaluate the possible effect of the agar on the sea urchins feeding behavior.

2.6.2. Sucrose diets

Three agar-based diets were prepared (Fig. 1): i) sucrose at the average natural concentration found in the plants (60 mg g DW^{-1}, SNC) (Brun et al., 2002, 2003a; Egea et al., 2018), ii) sucrose at half of the natural concentration found in the plants (30 mg g DW^{-1}, SLC) and iii) sucrose at 50% over the natural concentration found in the plants (90 mg g DW^{-1}, SHC). The agar blocks were prepared as described above (subsection 2.6.1).

2.6.3. Feeding experiments

Feeding experiments were carried out in a climatic chamber under controlled light and temperature conditions (18 °C). Sea urchins (115 individuals) were placed in several aquaria with natural seawater (20 L in each aquarium) in a closed flow-through system. The aquaria were illuminated by lamps with cool fluorescent tubes (T5 High Output Blau Aquaristic aquarium color extreme fluorescents) in a 8:16h (light: darkness) photoperiod. Aeration pits were placed in all aquaria to ensure adequate mixing of water and air. Sea urchins were allowed to acclimate to the aquarium conditions for a period of 86 h and during this period, the animals were fed with freshly collected *Ulva* sp. Prior to each assay experimental sea urchins were randomly separated from this large pool and starved for 24 h.

For each feeding assay three starved sea urchins and the corresponding diet (see below) were placed in an aquarium. After 1h, the sea-urchins were removed from the aquarium, placed in another tank and they were not used in further assays. Each agar block was weighed at the beginning and at the end of each assay and consumption rates were expressed as biomass consumed per individual per hour (g fresh weight $individual^{-1}$ h^{-1}).

Three types of feeding assays were conducted in triplicate: no-choice, two-choice, and three-choice feeding assays (Fig. 2). Individual agar blocks weighed 5.97 ± 0.08 g, 3.00 ± 0.07 g and 2.04 ± 0.08 g on average, depending on the diet trial (see below). Therefore, in the no-choice feeding assay, each aquarium contained 6 g of the agar blocks LC, LZ, BEC, BEZ, PNP, AC, SNC, SLC, and SHC (only one diet available in each aquarium) randomly located in the aquarium. In the two-choice feeding assay each aquarium contained at the same time the diets LC (3 g) and LZ (3 g). Finally, two three-choice feeding assay were carried out. In the first one each aquarium contained the diets BEC (2 g), BEZ (2 g) and PNP (2 g) at the same time. In the second one each aquarium contained the diets (SNC (2g), SLC (2 g) and SHC (2 g) at the same time. In all the assays the sea urchin:food ratio (3 sea urchins/6 g of food) was kept constant. Simultaneously, autogenic controls (i.e., agar-based diets but without including *P. lividus* in the aquarium) under the same experimental conditions were performed to account for potential changes in agar block weight not due to grazing (e.g. dissolving, fragmentation …). Results showed no significant changes in the weight of the autogenic agar blocks in any of the assays and therefore were not further considered in the analysis.

2.7. Statistical analyses

The differences between the initial and final wet weights of the different agar blocks were used to calculate the mass of material consumed (g $FW{\cdot}ind^{-1}{\cdot}h^{-1}$). For the choice feeding experiments, the amount of material consumed cannot be treated as independent due to the presence of multiple diets in a single aquarium (Peterson and Renaud, 1989). Thus, a multivariate Hotelling's T^2 test was used to determine significant differences between the consumption rate per individual and day (Roa, 1992; Manly, 1993; Prince et al., 2004), where the null hypothesis is based on no food preference by the consumer. Hotelling's trace multivariate statistic was computed with R v.3.0.2 (R Development Core Team 2013). Hotelling's T^2 was then calculated by multiplying the value for Hotelling's trace by (n -g), where n is the sample size (3) and g is the number of groups (2, 3), while the F value, degrees of freedom and level of significance remained the same in all tests (Crawley and Hyndes, 2007). For the no-choice feeding experiments a Tukey's post hoc analysis was applied when significant differences were found to assess significant differences between treatments. Prior to the analyses, normality (Shapiro-Wilk normality test) and homoscedasticity (Barlett test of homogeneity of variances test) of the data were checked.

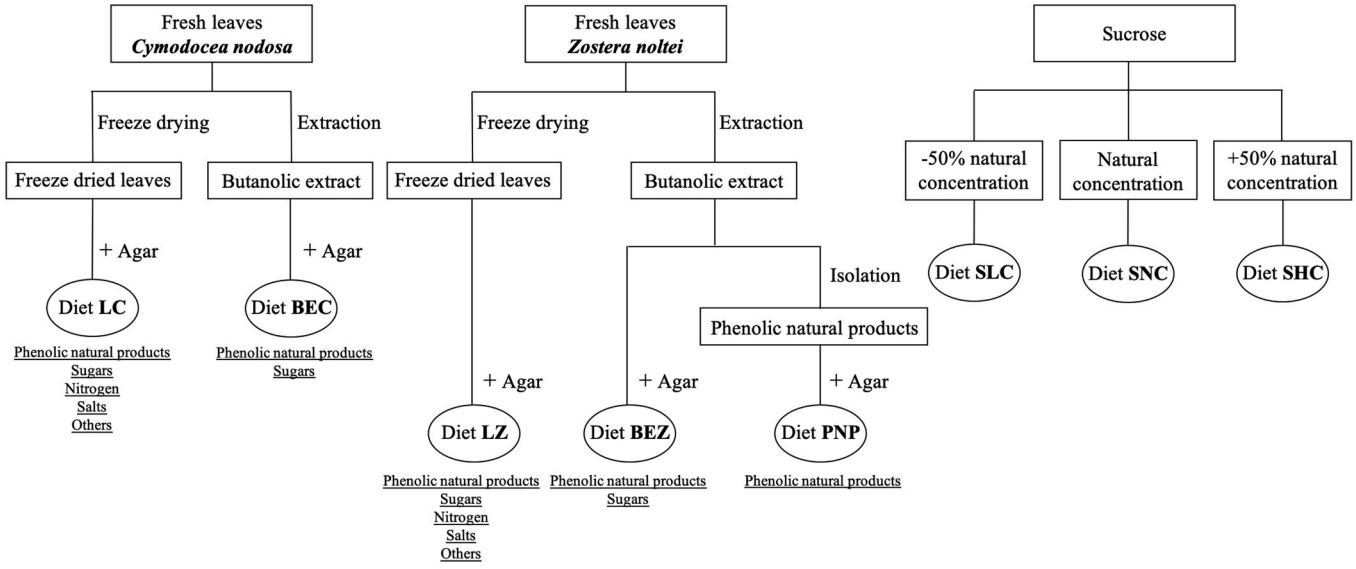

Fig. 1. Scheme of the agar-based artificial diets used in this study. LC: freeze-dried *C. nodosa* leaves; BEC: butanolic extract of *C. nodosa*. LZ: freeze-dried *Z. noltei* leaves; BEZ: butanolic extract of *Z. noltei;* PNP: phenolic natural products; SNC: sucrose at the natural concentration; SLC: sucrose at 50% of the natural concentration; SHC: sucrose content 50% higher than the natural concentration). The main components present in each diet after extraction and isolation are underlined.

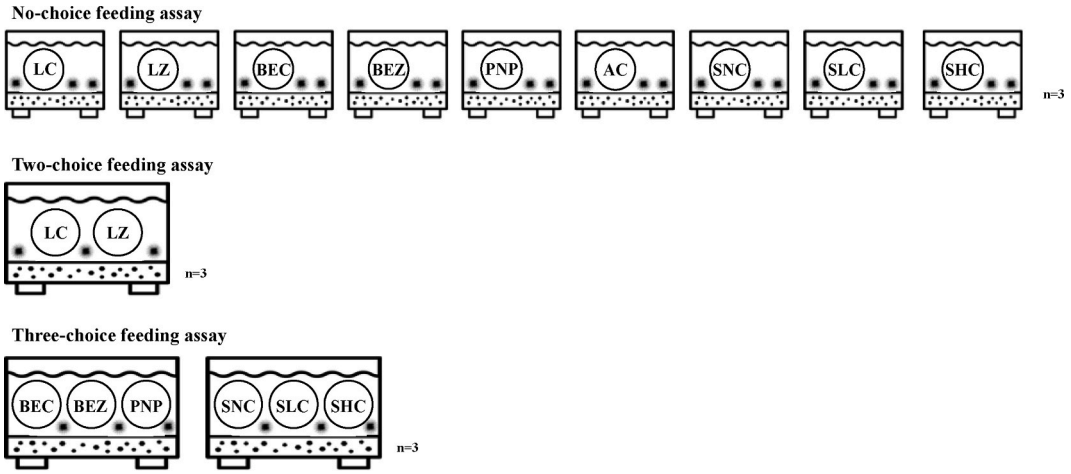

Fig. 2. Schematic representation of the experimental set-up. Circles represent the diets embedded in agar that were placed in each aquarium (LC: freeze-dried *C. nodosa* leaves; LZ: freeze-dried *Z. noltei* leaves; BEC: butanolic extract of *C. nodosa*; BEZ: butanolic extract of *Z. noltei*; PNP: phenolic natural products; SNC: sucrose at the natural concentration; SLC: sucrose at 50% of the natural concentration; SHC: sucrose content 50% higher than the natural concentration). Each feeding assay was performed by triplicate including the autogenic controls without sea urchins.

3. Results

3.1. Butanolic extracts of C. nodosa and Z. noltei for the feeding assays

The extraction of fresh leaves of *C. nodosa* and *Z. noltei* led to obtain butanolic extracts in yields of 5.6 ± 0.6 mg g FW^{-1} for *C. nodosa* and 23.4 ± 0.3 mg g FW^{-1} for *Z. noltei*. In the analysis by ^{1}H NMR (Fig. 3), the spectrum of the butanolic extract of *Z. noltei* showed signals between 6 and 8 ppm (marked with #) that indicated the presence of significant amounts of phenolic compounds in this extract. In particular, characteristic signals of rosmarinic acid, a typical metabolite of *Z. noltei*, were evident. However, the presence of phenolic compounds could not be detected from the ^{1}H NMR spectrum of the extract of *C. nodosa*. On the other hand, a common feature of the spectra of both extracts was the presence of signals attributable to sucrose, in particular the doublet at 5.38 ppm typical of the anomeric proton and various of the signals in the region 3.2–4.2 ppm (marked with *). Quantitative ^{1}H NMR analysis of the extracts allowed to determine that the concentration of sucrose per milligram of extract was 45% lower in the butanolic extract of *Z. noltei*.

3.2. Identification of the phenolic natural products of the extracts

The phenolic compounds of the extracts from leaves of *Z. noltei* were identified as rosmarinic acid (RA) and the five flavonoids apigenin-7-

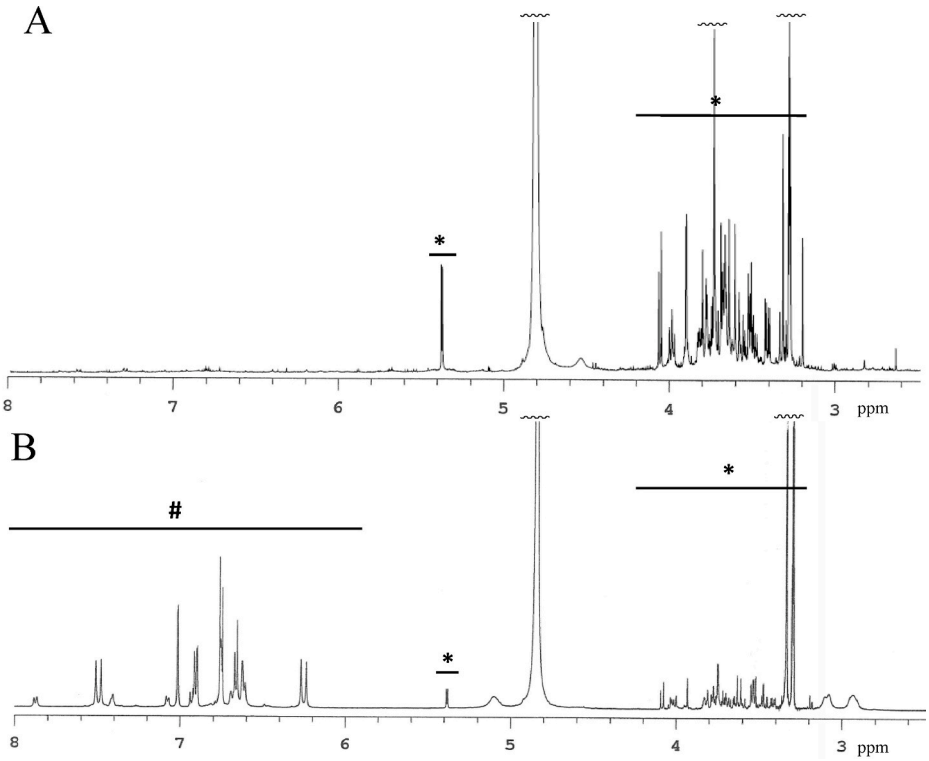

Fig. 3. ^{1}H-RMN spectra (CD_3OD, 500 MHz) of the butanolic extracts obtained from (**A**) leaves of *C. nodosa* and (**B**) leaves of *Z. noltei*. For each spectrum, the region containing signals distinctive of phenolic compounds is marked with hash (#) and the region with signals of sucrose is marked with asterisk (*).

sulfate (APS), lutein-7-sulfate (LS), diosmetin-7-sulfate (DS), acacetin-7-sulfate (ACS), and apigenin-7-*O*-glucoside (APG) (Fig. 4). The quantitative analysis of the extracts determined that rosmarinic acid was the major phenolic compound in the leaves of *Z. noltei*, with a concentration of 12.85 ± 0.40 mg·FW^{-1} and that the sum of the five flavonoids represented a total of 7.05 ± 0.74 mg FW^{-1}.

3.3. Feeding assays

3.3.1. Diets based on freeze-dried leaves, extracts, and phenolic natural products

In the no choice feeding assay, *Paracentrotus lividus* showed a differential consumption rate depending on the diet (i.e. LC, LZ, BEC, BEZ, PNP, AC) (Fig. 5A, Table 1). The most consumed diets were those including freeze-dried leaves of *C. nodosa* (LC) and *Z. noltei* (LZ), which contained all the nutritional (nitrogen, sugars, etc) and chemical traits (phenolic natural products). Consumption rates significantly decreased in those diets consisting on the butanolic extracts (BEC and BEZ diets), especially for that derived from *Z. noltei*, while a null consumption rate was observed when the diet was composed of phenolic natural products (PNP) (Fig. 5A). Agar blocks (AC) consumption was trivial but significantly higher to those values recorded in PNP (Fig. 5A).

In the two-choice assay, sea urchins consumed preferentially the diet composed of freeze-dried leaves of *C. nodosa* over *Z. noltei* leaves (Fig. 5B, Table 1). The consumption rate per individual in the two-choice assay, that is, the sum of the consumption rates recorded for both diets, was not significantly different from those values recorded in the no-choice feeding assay (0.799 ± 0.064 g fresh weight individual^{-1} h^{-1} for LC or LZ in the no-choice and 0.820 ± 0.024 g fresh weight individual^{-1} h^{-1} for two-choice assay) (Fig. 5B).

In the three-choice assay, sea urchins showed the same preferential feeding behavior previously recorded in the no-choice assay, with significantly higher consumption rates of *C. nodosa* butanolic extract, and a residual consumption over phenolic natural products (Fig. 5C, Table 1). Moreover, the consumption rates per individual were lower than those observed in the no-choice and two-choice feeding assays (Fig. 5C).

3.3.2. Diets based on sucrose

Sucrose concentration had a significant effect on the sea urchin feeding behavior, since consumption rates was circa ten times higher than control agar consumption in the no-choice assay independently of sucrose concentration (Fig. 6A, Table 2). In the three-choice assay, *P. lividus* significantly preferred those diets containing higher concentrations of sucrose (Fig. 6B, Table 2), although the consumption rate per individual in the three-choice assay was not significantly different from those values recorded in the no-choice feeding assay (0.964 ± 0.037 g fresh weight individual^{-1} h^{-1} in average for SLC, SNC, and SHC in the no-choice and 1.042 ± 0.041 g fresh weight individual^{-1} h^{-1} for three-choice assay).

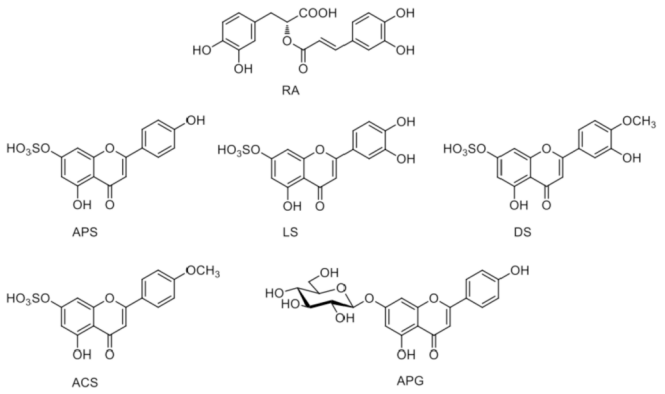

Fig. 4. Chemical structures of the phenolic natural products of the leaves of *Z. noltei*. (RA) rosmarinic acid; (APS) apigenin-7-sulfate; (LS) lutein-7-sulfate; (DS) diosmetin-7-sulfate; (ACS) acacetin-7-sulfate; (APG) apigenin-7-*O*-glucoside.

4. Discussion

This work has shown that the feeding behavior of the generalist herbivore *P. lividus* over the two template seagrass species *C. nodosa* and *Z. noltei*, once overlooked the structural, morphological, and biomechanical traits, is determined by nutritional properties. The presence of phenolic compounds has a minimal effect on their feeding behavior. In addition, this study has demonstrated for the first time the deterrent properties of the mixture of rosmarinic acid and sulphated flavonoids produced by *Z. noltei*, which are able to reduce the attractive effect of sugars in the feeding preference of this herbivore.

Is it largely known that the generalist herbivore *P. lividus* can alter its feeding behavior depending on the palatability of plant tissues (Jiménez-Ramos et al., 2017, 2018a,b; Vergés et al., 2007a,b, 2011). However in others studies no feeding preference was observed when different food sources were available (Cacabelos et al., 2010; Cardoso et al., 2020). In the studies where sea urchins showed some active behavioral choice, animals used to forage over those tissues containing lower structural defenses (Vergés et al., 2007b, 2011), higher nutritional properties (Jiménez-Ramos et al., 2018a) and lower chemical defenses (Vergés et al., 2007a; Rodríguez et al., 2017).

In the no-choice feeding assay of our study, *P. lividus* preferred diets based on freeze-dried leaves of *Cymodocea nodosa* (LC) and *Zostera noltei* (LZ). On the other hand the consumption of butanolic extracts was lower, and null consumption rate was recorded in the case of the diet based on phenolic compounds. Freeze-dried leaves contain all the nutritional traits of the seagrass tissues (metabolites rich in nitrogen, lipids, organic matter, sugars, etc), but also natural products (mainly phenolic compounds) and salts. Therefore, the inclusion of freeze-dried leaves in the blocks of agar enhanced the nutritional quality of the diet, since consumption over control agar blocks was trivial in all the treatments. This is in agreement with previous studies showing the preference of sea urchins for foods with higher nutritional quality (Vergés et al., 2007b; Prado and Heck, 2011; Rodríguez et al., 2017; Jiménez-Ramos et al., 2018a) and, in particular, for those diets composed of a high proportion of nitrogen rich compounds (e.g. proteins) (Zieman et al., 1984; Fernandez, 1997; Fernandez and Boudouresque, 1998; Hammer et al., 2006). During the preparation of the butanolic extracts of the leaves most of the nutritional traits (compounds rich in nitrogen, lipids, carbohydrates etc) were eliminated, and thus the butanolic extracts are mainly composed of phenolic natural products and/or some amounts of sugars, as shown in the ^{1}H-RMN spectra of the extracts of both species. The decrease of the nutritional quality of these diets (i.e. BEC and BEZ) resulted in a clear and significant reduction of the consumption rates, which was more pronounced in the case of the BEZ diet, fully in line with the preference for the diets with high nutritional value above mentioned.

The large differences in the consumption rates between BEC and BEZ diets when supplied together can be explained by the composition of these extracts. We observed that the extract of *Z. noltei* possessed high amounts of natural phenolic compounds, clearly detected in the ^{1}H-NMR spectrum, which largely differed from the extract of *C. nodosa*, where natural phenolic compounds were almost absent. This result is in agreement with previous studies that have shown significant qualitative and quantitative differences in the phenolic content between *C. nodosa* and *Z. noltei* inhabiting the Bay of Cadiz, with the former species containing much lower concentrations of phenolic natural products (up to 0.7 mg g DW^{-1}) (Jiménez-Ramos et al., 2017) than the latter (up to 92 mg g DW^{-1}) (Manck et al., 2017). On the other hand, we found that the extracts of both species contained sucrose, thus confirming that the extraction procedures used to extract phenolic natural products also lead

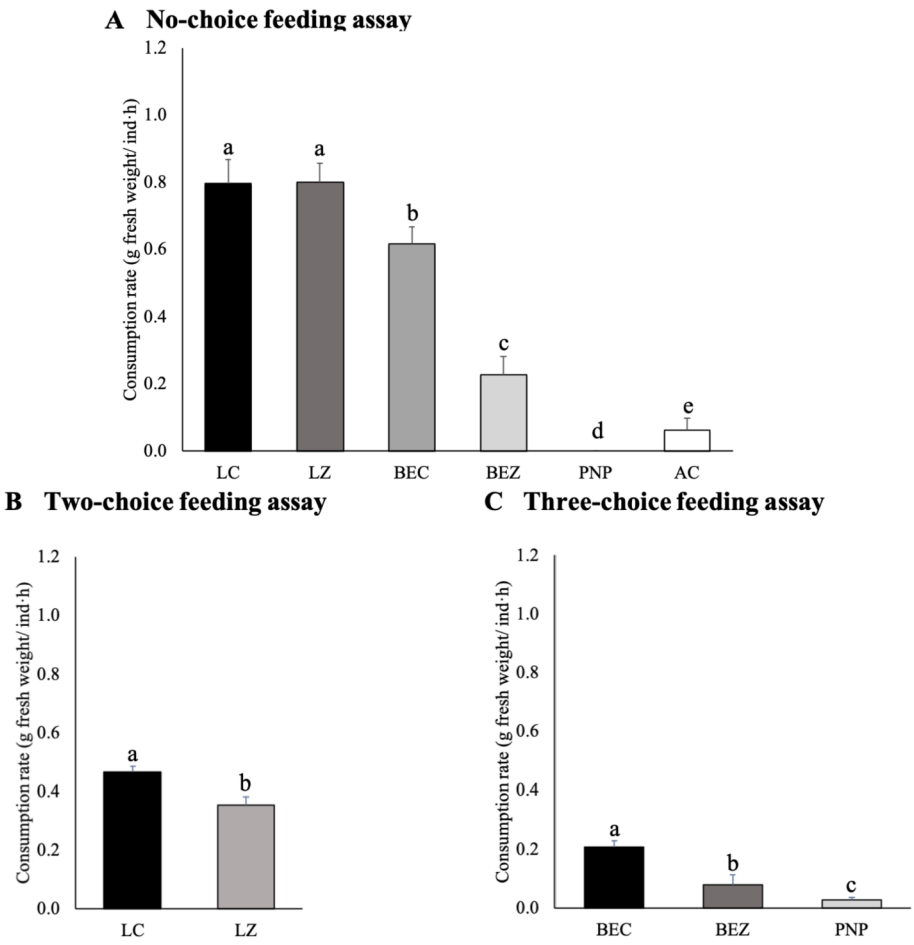

Fig. 5. Results of the feeding assays examining different agar-based diets. (A) No-choice feeding assay; (B) Two-choice feeding assay; (C) Three-choice feeding assay. LC: freeze-dried leaves of *C. nodosa*; LZ: freeze-dried leaves of *Z. noltei*; BEC: butanolic extract of *C. nodosa* leaves; BEZ: butanolic extract of *Z. noltei* leaves; PNP: phenolic natural products; AC: agar control. Data shown are mean ± SE. All the treatments were done by triplicate. Inset letters indicate significant differences at $p < 0.05$.

Table 1
Results of the one-way ANOVA examining the differences in the consumption rates of *P. lividus* over different agar-based diets in the no-choice feeding assay. Hotelling multivariate test was used to examine differences in consumption rates in the two- and three-choice feeding assays. Bold numbers indicate significant differences at $p < 0.05$.

One-way ANOVA		df	MS	F	*P*
No-choice feeding assay		5	1.71	58.41	**< 0.01**
Hotelling's Test (T^2)		**df**	**T^2**	**F**	***P***
Two-choice feeding assays	LC *vs* LZ	3	7.305	2.793	**0.009**
Three-choice feeding assays	BEC *vs* BEZ *vs* PNP	3	8.751	3.621	**0.031**

to recover a portion of the soluble carbohydrates of the plant (Dai and Mumper, 2010). The potential influence of these sugars in the outcome of feeding assays using extracts that are supposed to contain only phenolic compounds, has never been analyzed. Noteworthy, our study has demonstrated that when sucrose-based diets are offered to *P. lividus*, the consumption rate is enhanced and that is directly related to sucrose concentration. This finding seems logical since herbivores use to maximize their foraging time trying to consume aliments with high nutritional and energetic values as stated by the theory of optimal forage (Vadas, 1977; McClintock, 1986; Schlosser et al., 2005). However, it is important to state that although sugar content seems to be a trait affecting to the active behavioral choice of *P. lividus*, it seems to be subordinated to the presence of compounds rich in nitrogen (i.e. freeze-dried diets). The importance of the balance in the diet between protein:carbohydrate in the growth and gamete development in sea urchins has been previously studied, and indicated that those diets based in high protein and low carbohydrate levels were the most optimal to promote the development of gonads and the growth of the individuals (Fernandez, 1997; Fernandez and Boudouresque, 1998; Hammer et al., 2006). The extract of *Z. noltei* was lower in sucrose but the extracts of *C. nodosa* and *Z. noltei* were included in the agar diets at the same concentrations that they were obtained from fresh plants (i.e. a concentration four-fold higher for *Z. noltei*). Taken together, this yielded that the BEZ diet contained more sugars than the BEC and, therefore, the former diet should be potentially more attractive for *P. lividus*. However, we recorded a lower consumption over BEZ diets, which must be due to the higher concentration of phenolic compounds present in the extract of *Z. noltei*, whose deterrent activity is able to surpass the attractive effect of sugars. This proposal could find further support in next studies assaying mixtures of phenolic compounds and sugars at different concentrations.

The diets consisting of phenolic compounds at the naturally occurring concentration were not consumed when offered to sea urchins in the no-choice assays, and only marginally consumed in the three-choice assays, which agrees with the deterrent role that has been ascribed to this class of compounds in some previous studies (Harrison, 1982; Goecker et al., 2005; Vergés et al., 2011, 2018). However, this deterrent effect was of limited importance in our study, since *P. lividus* consumed diets with phenolic compounds when all nutritional properties were present (i.e., freeze-dried leaves). This finding is in agreement with some studies that have recorded that *P. lividus* consumed macrophyte species that carried phenolic and other chemical compounds (Frantzis and Grémare, 1992; Vergés et al., 2007a,b, 2011; Rodríguez et al., 2017; Cardoso et al., 2020). However in other cases, consumption of macrophytes with toxic or deterrent compounds produced a reduction in their consumption rate or even sublethal effects (e.g., loss of spines,

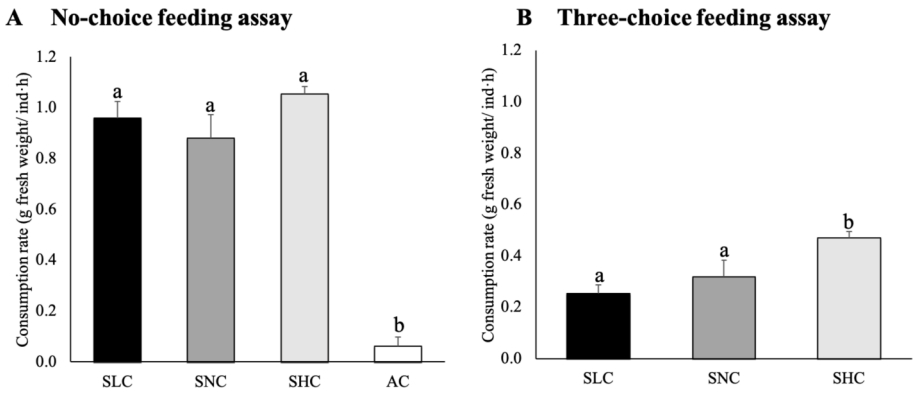

Fig. 6. Results of the feeding assays examining different concentrations of sucrose in agar-based diets. (A) No-choice feeding assay; (B) Three-choice feeding assay. SLC: sucrose at 50% of the natural concentration; SNC: sucrose at the natural concentration; SHC: sucrose content 50% higher than the natural concentration; AC: agar control. Data are showed as mean ± SE. All the treatments were done by triplicate. Inset letters indicate significant differences at $p < 0.05$.

Table 2
Results of the one-way ANOVA examining the differences in the consumption rates of *P. lividus* over different concentrations of sucrose in agar-based diets in the no-choice feeding assay. Hotteling multivariate test was used to examine differences in consumption rates between the different concentrations of sucrose in the three-choice feeding assay. Bold numbers indicate significant differences at $p < 0.05$.

One-way ANOVA	Df	MS	*F*	*p*
No-choice feeding assay	3	0.7806	154.21	**< 0.0001**
Hotteling's test	**Df**	**T^2**	***F***	***p***
Three-choice feeding assay	3	6.9453	2.9534	**0.043**

antioxidant response; Boudouresque et al., 1996; Tejada et al., 2013). In spite of this moderate contribution of the phenolic natural products to the feeding behavior of *P. lividus*, the high phenolic content of BEZ diets also supports the lower preference for this diet compared to BEC diet, in spite of the higher sucrose content in BEZ diets. Moreover, only few studies have analyzed the direct effects of seagrass extracts on the feeding behavior of herbivores (Goecker et al., 2005; Vergés et al., 2007a), although none of these studies identified the natural products present in these extracts. The isolation, characterization, and quantification of the natural products in the extracts is necessary to understand which chemical compound (or combination of compounds) is behind the deterrent activity (Heck and Valentine, 2006; Sieg and Kubanek, 2013), since phenolic natural products in seagrasses comprise a large number of chemical compounds with different functions (i.e. deterrent, antifouling, antimicrobial, uv protector, etc) (Sieg and Kubanek, 2013; Subhashini et al., 2013; Zidorn, 2016). In this study, five flavonoids were isolated from *Z. noltei*, along with rosmarinic acid as the major compound. Although these natural products are not new, as they had previously been isolated from *Z. noltei* (Manck et al., 2017; Enerstvedt et al., 2017; Grignon-Dubois and Rezzonico, 2018), this is the first time they have been linked to their likely deterrent function. In the marine environment rosmarinic acid has only been found in *Zostera* species (Zidorn, 2016; Manck et al., 2017; Grignon-Dubois and Rezzonico, 2018; Papazian et al., 2019), while this phenolic acid is widely distributed among terrestrial plants (Petersen et al., 2009; Petersen, 2013). The ecological significance of rosmarinic acid has been poorly defined, with a few studies indicating that in terrestrial plants this acid is a constitutive, in some instances inducible, defense against pathogens (Bais et al., 2002; Walker et al., 2004; Widmer and Laurent, 2006) and herbivores (Khan et al., 2019; Simmonds et al., 2019). On the other hand, sulphated flavonoids are a reduced group of flavonoids that, besides from *Zostera* and *Thalassia* genera (Zidorn, 2016; Manck et al., 2017; Enerstvedt et al., 2017; Grignon-Dubois and Rezzonico, 2018; Papazian et al., 2019), have been isolated mainly from some terrestrial plants growing near aquatic areas rich in salts (Teles et al., 2018). In fact, the presence of sulfate groups in these metabolites is considered an ecological adaption to aquatic environments (Tomás-Barberán et al., 1987). The role of rosmarinic acid and sulphated flavonoids as chemical defences in seagrasses is scarcely known, and only recently some authors (Guan et al., 2017; Papazian et al., 2019) have shown that rosmarinic acid, and to a lesser extent the flavonoids, play a role in the prevention of microbial settlement on *Z. marina* leaves. Herein, we have shown for the first time that rosmarinic acid and the sulphated flavonoids present in *Z. noltei* exhibited deterrent properties and played a role in the defense of this species against herbivores.

When *P. lividus* was supplied with different food sources (i.e. two- and three-choice feeding assays), displayed a clear active behavioral choice, which is required to state the existence of a preference in the feeding behavior (Singer, 2000). LC diet was slightly preferred over LZ diet, while BEC diet was preferentially consumed over BEZ and PNP diets. Taking into account the aforementioned regarding to the importance of nutritional traits, natural products presence, and sugars content in the feeding behavior, these results clearly support the existence of a subordinate hierarchy in those traits determining the active feeding behavior of *P. lividus* over these seagrass species, with nutritional traits (i.e. nitrogen rich compounds, lipids, etc) as the main trait, followed by phenolic natural products and finally sugar content. Moreover, it is important to note that we did not record a compensatory feeding behavior (Valentine and Heck, 2001; Cruz-Rivera and Hay, 2000) in our experimental assays, since those diets with lower nutritional values were not more consumed, and accumulated consumption rates between no-choice and choice assays were similar. Maybe, our experimental time (1 h) was not long enough to detect such phenomenon, and sea urchins spent most of the experimental time foraging between the different diets, which finally reduced the effective feeding time. Some authors using longer experimental times (i.e. weeks and months) and providing food *ad libitum*, recorded a compensatory feeding behavior in this species (Mazzella et al., 1992; Cruz-Rivera and Hay, 2000; Valentine and Heck, 2001), but other authors were unable to find such response (Lawrence et al., 1989; Jiménez-Ramos et al., 2018b), maybe as a result of the absorption efficiency plasticity displayed by sea urchins (Lawrence et al., 1989). Regardless of the existence of this compensatory feeding response, feeding rates were within the range observed in previous studies using such experimental design (Jiménez-Ramos et al., 2018a,b).

In summary, this study demonstrated that the generalist herbivore *P. lividus* preferentially fed on those diets with higher nutrient content (e.g. nitrogen, sugar) and lower chemical defenses. In addition, for the first time, the significance of sugar concentration as a feeding attractant in seagrasses was demonstrated, while the role played by rosmarinic acid and sulphated flavonoids in the defense against herbivores was also

highlighted. Since all these factors (nitrogen, sugar and phenolic natural products content) use to covariate in response to environmental stressors (e.g. light, nutrients, temperature, etc), our results may help to explain heterogeneity and changes in herbivory intensity in seagrass communities. Moreover, the study of the attractiveness of nitrogen and/or sugar contents versus the deterrent properties of phenolic compounds deserve future research efforts, in order to find those ratio thresholds that may enhance or reduce the loss of tissues due to herbivore consumption.

Authors statement

I. Casal-Porras: conceptualization, investigation, writing, review and editing; R. Jiménez-Ramos: investigation; E. Zubía: conceptualization, investigation, supervision, writing, review and editing; F.G. Brun: conceptualization, investigation, supervision, funding acquisition, writing, review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2021.107466.

References

Agrawal, A.A., 2011. Current trends in the evolutionary ecology of plant defence. Funct. Ecol. 25, 420–432. https://doi.org/10.1111/j.1365-2435.2010.01796.x.

Agrawal, A.A., Fishbein, M., 2006. Plant defense syndromes. In: Ecology 87, pp. S132–S149. https://doi.org/10.1890/0012-9658(2006)87[132:PDS]2.0.CO;2.

Appel, H.M., 1993. Phenolics in ecological interactions: the importance of oxidation. J. Chem. Ecol. 19, 1521–1552. https://doi.org/10.1007/BF00984895.

Arnold, T., Mealey, C., Leahey, H., Miller, A.W., Hall-Spencer, J.M., Milazzo, M., Maers, K., 2012. Ocean acidification and the loss of phenolic substances in marine plants. PloS One 7, e35107. https://doi.org/10.1371/journal.pone.0035107.

Bais, H.P., Walker, T.S., Schweizer, H.P., Vivanco, J.M., 2002. Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of *Ocinum basilicum*. Plant Physiol. Biochem. 40, 983–995. https://doi.org/10.1016/S0981-9428(02)01460-2.

Ballorain, K., Ciccione, S., Bourjea, J., Grizel, H., Enstipp, M., Georges, J.Y., 2010. Habitat use of a multispecific seagrass meadow by green turtles *Chelonia mydas* at Mayotte Island. Mar. Biol. 157, 2581–2590. https://doi.org/10.1007/s00227-010-1520-7.

Boudouresque, C.F., Lemée, R., Mari, X., Meinesz, A., 1996. The invasive alga *Caulerpa taxifolia* is not a suitable diet for the sea urchin *Paracentrotus lividus*. Aquat. Bot. 53, 245–250. https://doi.org/10.1016/0304-3770(96)01021-2.

Boudouresque, C.F., Verlaque, M., 2001. Ecology of *Paracentrotus lividus*. Dev. Aquacult. Fish. Sci. 32, 177–216. https://doi.org/10.1016/S0167-9309(01)80013-2.

Brun, F.G., Hernández, I., Vergara, J.J., Peralta, G., Pérez-Lloréns, J.L., 2002. Assessing the toxicity of ammonium pulses to the survival and growth of *Zostera noltii*. Mar. Ecol. Prog. Ser. 225, 177–187. https://doi.org/10.3354/meps225177.

Brun, F.G., Pérez-Lloréns, J.L., Hernández, I., Vergara, J.J., 2003a. Patch distribution and within-patch dynamics of the seagrass *Zostera noltii* hornem. In los toruños salt-marsh, Cádiz Bay, natural park, Spain. Bot. Mar. 46, 513–524. https://doi.org/10.1515/BOT.2003.053.

Brun, F.G., Vergara, J.J., Navarro, G., Hernández, I., Pérez-Lloréns, J.L., 2003b. Effect of shading by *Ulva rigida* canopies on growth and carbon balance of the seagrass *Zostera noltii*. Mar. Ecol. Prog. Ser. 265, 85–96. https://doi.org/10.3354/meps265085.

Buchsbaum, R.N., Short, F.T., Cheney, D.P., 1990. Phenolic-nitrogen interaction in eelgrass, *Zostera marina* L.: possible implication for disease resistance. Aquat. Bot. 37, 291–297.

Buchsbaum, R., Valiela, I., 1987. Variability in the chemistry of estuarine plants and its effect on feeding by Canada geese. Oecologia 73, 146–153. https://doi.org/10.1007/BF00376991.

Cacabelos, E., Olabarria, C., Incera, M., Troncoso, J.S., 2010. Do grazers prefer invasive seaweeds? J. Exp. Mar. Biol. Ecol. 393, 182–187. https://doi.org/10.1016/j.jembe.2010.07.024.

Cardoso, A.C., Arenas, F., Sousa-Pinto, I., Barreiro, A., Franco, J.N., 2020. Sea urchin grazing preferences on native and non-native macroalgae. Ecol. Indicat. 111, 106046. https://doi.org/10.1016/j.ecolind.2019.106046.

Carmona, D., Lajeunesse, M.J., Johnson, M.T., 2011. Plant traits that predict resistance to herbivores. Funct. Ecol. 25, 358–367. https://doi.org/10.1111/j.1365-2435.2010.01794.x.

Cebrián, J., Duarte, C.M., Marbà, N., 1996. Herbivory on the seagrass *Cymodocea nodosa* (Ucria) ascherson in contrasting Spanish Mediterranean habitats. J. Exp. Mar. Biol. Ecol. 204, 103–111.

Chapin, F.S., Matson, P.A., Vitousek, P.M., 2011. Trophic dynamics. In: Principles of Terrestrial Ecosystem Ecology. Springer, New York, NY, pp. 297–320.

Coley, P.D., Barone, J.A., 1996. Herbivory and plant defenses in tropical forests. Annu. Rev. Ecol. Systemat. 27, 305–335. https://doi.org/10.1146/annurev.ecolsys.27.1.305.

Crawley, K.R., Hyndes, G.A., 2007. The role of different types of detached macrophytes in the food and habitat choice of a surf-zone inhabiting amphipod. Mar. Biol. 151, 1433–1443. https://doi.org/10.1007/s00227-006-0581-0.

Cruz-Rivera, E., Hay, M.E., 2000. Can quantity replace quality? Food choice, compensatory feeding. and fitness of marine mesograzers 201–219. https://doi.org/10.1890/0012-9658(2000)081[0201:CQRQFC]2.0.CO;2. Ecology 81.

Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313–7352. https://doi.org/10.3390/molecules15107313.

De los Santos, C.B., Arenas, F., Neuparth, T., Santos, M.M., 2019. Interaction of short-term copper pollution and ocean acidification in seagrass ecosystems: toxicity, bioconcentration and dietary transfer. Mar. Pollut. Bull. 142, 155–163. https://doi.org/10.1016/j.marpolbul.2019.03.034.

De los Santos, C.B., Brun, F.G., Onoda, Y., Cambridge, M.L., Bouma, T.J., Vergara, J.J., Pérez-Lloréns, J.L., 2012. Leaf-fracture properties correlated with nutritional traits in nine Australian seagrass species: implications for susceptibility to herbivory. Mar. Ecol. Prog. Ser. 458, 89–102. https://doi.org/10.3354/meps09757.

De los Santos, C.B., Brun, F.G., Vergara, J.J., Pérez-Lloréns, J.L., 2013. New aspect in seagrass acclimation: leaf mechanical properties vary spatially and seasonally in the temperate species *Cymodocea nodosa* Ucria (Ascherson). Mar. Biol. 160, 1083–1093. https://doi.org/10.1007/s00227-013-2200-1.

De los Santos, C.B., Onoda, Y., Vergara, J.J., Pérez-Lloréns, J.L., Bouma, T.J., La Nafie, Y. A., Cambridge, M.L., Brun, F.G., 2016. A comprehensive analysis of mechanical and morphological traits in temperate and tropical seagrass species. Mar. Ecol. Prog. Ser. 551, 81–94. https://doi.org/10.3354/meps11717.

Duffy, J.E., Hay, M.E., 1990. Seaweed adaptations to herbivory. Bioscience 40, 368–375. https://doi.org/10.2307/1311214.

Egea, L.G., Jiménez-Ramos, R., Vergara, J.J., Hernández, I., Brun, F.G., 2018. Interactive effect of temperature, acidification and ammonium enrichment on the seagrass *Cymodocea nodosa*. Mar. Pollut. Bull. 134, 14–26. https://doi.org/10.1016/j.marpolbul.2018.02.029.

Enerstvedt, K.H., Lundberg, A., SjØtun, I.K., Fadnes, P., Jordheim, M., 2017. Characterization and seasonal variation of individual flavonoids in *Zostera marina* and *Zostera noltii* from Norwegian coastal waters. Biochem. Systemat. Ecol. 74, 42–50. https://doi.org/10.3390/molecules23010016.

Fernandez, C., 1997. Effect of diet on the biochemical composition of *Paracentrotus lividus* (Echinodermata: Echinoidea) under natural and rearing conditions (effect of diet on biochemical composition of urchins). Comp. Biochem. Physiol. 118, 1377–1384.

Fernandez, C., Boudouresque, C.F., 1998. In: Mooi, R., Telford, M. (Eds.), Evaluating Artificial Diet for Small *Paracentrotus lividus* (Echinodermata: Echinoidea). Ln, Echinoderms, San Francisco. Balkema, Rotterdam, pp. 651–656.

Fernández, C., Ferrat, L., Pergent, G., Pasqualini, V., 2012. Sea urchin-seagrasses interactions: trophic links in a benthic ecosystem from a coastal lagoon. Hydrobiologia 699, 21–33.

Frantzis, A., Grémare, A., 1992. Ingestion, absorption, and growth rates of *Paracentrotus lividus* (Echinodermata: echinoidea) fed different macrophytes. Mar. Ecol. Prog. Ser. 95, 169–183.

Fürstenberg-Hägg, J., Zagrobelny, M., Bak, S., 2013. Plant defense against insect herbivores. Int. J. Mol. Sci. 14, 10242–10297. https://doi.org/10.3390/ijms140510242.

Gera, A., Pagès, J.F., Romero, J., Alcoverro, T., 2013. Combined effects of fragmentation and herbivory on *Posidonia oceanica* seagrass ecosystems. J. Ecol. 101, 1053–1061. https://doi.org/10.1111/1365-2745.12109.

Goecker, M.E., Heck Jr., K.L., Valentine, J.F., 2005. Effects of nitrogen concentrations in turtlegrass *Thalassia testudinum* on consumption by the bucktooth parrotfish *Sparisoma radians*. Mar. Ecol. Prog. Ser. 286, 239–248. https://doi.org/10.3354/meps286239.

Gong, B., Zhang, G., 2014. Interactions between plants and herbivores: a review of plant defense. Acta Ecol. Sin. 34, 325–336. https://doi.org/10.1016/j.chnaes.2013.07.010.

Grignon-Dubois, M., Rezzonico, B., 2018. Phenolic chemistry of the seagrass *Zostera noltei* Hornem. Part 1: first evidence of three infraspecific flavonoid chemotypes in three distinctive geographical regions. Phytochemistry 146, 91–101. https://doi.org/10.1016/j.phytochem.2017.12.006.

Guan, C., Parrot, D., Wiese, J., Sönnichsen, F.D., Saha, M., Tasdemir, D., Weinberger, F., 2017. Identification of rosmarinic acid and sulfated flavonoids as inhibitors of microfouling on the surface of eelgrass *Zostera marina*. Biofouling 33, 867–880. https://doi.org/10.1080/08927014.2017.1383399.

Hammer, H., Hammer, B., Watts, S., Lawrence, A., Lawrence, J., 2006. The effect of dietary protein and carbohydrate concentration on the biochemical composition and gametogenic condition of the sea urchin *Lytechinus variegatus*. J. Exp. Mar. Biol. Ecol. 334, 109–121. https://doi.org/10.1016/j.jembe.2006.01.015.

Harrison, P.G., 1982. Control of microbial growth and of amphipod grazing by water-soluble compounds from leaves of *Zostera marina*. Mar. Biol. 67, 225–230. https://doi.org/10.1007/BF00401288.

Heck Jr., K.L., Valentine, J.F., 2006. Plant–herbivore interactions in seagrass meadows. J. Exp. Mar. Biol. Ecol. 330, 420–436. https://doi.org/10.1126/science.1059199.

Hemminga, M.A., Duarte, C.M., 2000. Seagrass Ecology. Cambridge University Press, Cambridge, UK.

Hernandez, A.L.M., Van Tussenbroek, B.I., 2014. Patch dynamics and species shifts in seagrass communities under moderate and high grazing pressure by green sea turtles. Mar. Ecol. Prog. Ser. 517, 143–157. https://doi.org/10.3354/meps11068.

Jackson, J.B., Kirby, M.X., Berger, W.H., Bjorndal, K.A., Botsford, L.W., Bourque, B.J., Bradbury, R.H., Cooke, R., Erlandson, J., Estes, J.A., Hughes, T.P., Kidwell, S., Lange, C.B., Lenihan, H.S., Pandolfi, J.M., Peterson, C.H., Steneck, R.S., Tegner, M.J., Warner, R.R., 2001. Historical overfishing and the recent collapse of coastal ecosystems. Science 293, 629–637. https://doi.org/10.1126/science.1059199.

Jakobsson-Thor, S., Toth, G.B., Brakel, J., Bockelmann, A.C., Pavia, H., 2018. Seagrass wasting disease varies with salinity and depth in natural *Zostera marina* populations. Mar. Ecol. Prog. Ser. 587, 105–115. https://doi.org/10.3354/meps12406.

Jiménez-Ramos, R., Brun, F.G., Egea, L.G., Vergara, J.J., 2018a. Food choice effects on herbivory: Intra-specific seagrass palatability and inter-specific macrophyte palatability in seagrass communities. Estuar. Coast Shelf Sci. 204, 31–39. https://doi.org/10.1016/j.ecss.2018.02.016.

Jiménez-Ramos, R., Egea, L.G., Ortega, M.J., Hernández, I., Vergara, J.J., Brun, F.G., 2017. Global and local disturbances interact to modify seagrass palatability. PloS One 12, e0183256. https://doi.org/10.1371/journal.pone.0183256.

Jiménez-Ramos, R., Egea, L.G., Vergara, J.J., Brun, F.G., 2018b. Nutrient load and epiphytes are drivers of increased herbivory in seagrass communities. Mar. Ecol. Prog. Ser. 599, 49–64. https://doi.org/10.3354/meps12622.

Johnson, M.T., 2011. Evolutionary ecology of plant defences against herbivores. Funct. Ecol. 25, 305–311. https://doi.org/10.1111/j.1365-2435.2011.01838.x.

Kelkar, N., Arthur, R., Marba, N., Alcoverro, T., 2013. Green turtle herbivory dominates the fate of seagrass primary production in the Lakshadweep islands (Indian Ocean). Mar. Ecol. Prog. Ser. 485, 235–243. https://doi.org/10.3354/meps10406.

Khan, S., Taning, C.N.T., Bonneure, E., Mangelinckx, S., Smagghe, G., Ahmad, R., Fatima, N., Asif, M., Shah, M.M., 2019. Bioactivity-guided isolation of rosmarinic acid as the principle bioactive compound from the butanol extract of *Isodon rugosus* against the pea aphid, *Acyrthosiphon pisum*. PloS One 14, e0215048. https://doi.org/10.1371/journal. pone.0215048.

La Nafie, Y.A., De los Santos, C.B., Brun, F.G., Mashoreng, S., van Katwijk, M.M., Bouma, T.J., 2013. Biomechanical response of two fast-growing tropical seagrass species subjected to in situ shading and sediment fertilization. J. Exp. Mar. Biol. Ecol. 446, 186–193. https://doi.org/10.1016/j.jembe.2013.05.020.

La Nafie, Y.A., De Los Santos, C.B., Brun, F.G., Van Katwijk, M.M., Bouma, T.J., 2012. Waves and high nutrient loads jointly decrease survival and separately affect morphological and biomechanical properties in the seagrass *Zostera noltii*. Limnol. Oceanogr. 57, 1664–1672. https://doi.org/10.4319/lo.2012.57.6.1664.

Lawrence, J., Régis, M.B., Delmas, P., Gras, G., Klinger, T., 1989. The effect of quality of food on feeding and digestion in *Paracentrotus lividus* (Lamarck) (Echinodermata: echinoidea). Mar. Freshw. Behav. Physiol. 15, 137–144. https://doi.org/10.1080/10236248909378724.

Lefebvre, L.W., Reid, J.P., Kenworthy, W.J., Powell, J.A., 2000. Characterizing manatee habitat use and seagrass grazing in Florida and Puerto Rico: implications for conservation and management. Pac. Conserv. Biol. 5, 289–298. https://doi.org/10.1071/PC000289.

Manck, L., Quintana, E., Suárez, R., Brun, F.G., Hernández, I., Ortega, M.J., Zubía, E., 2017. Profiling of phenolic natural products in the seagrass *Zostera noltei* by UPLC-MS. Nat. Prod. Commun. 12, 687–690. https://doi.org/10.1177/1934578X1701200511.

Manly, B.F.J., 1993. Comments on design and analysis of multiple-choice feeding reference experiments. Oecologia 93, 149–152.

Mariani, S., Alcoverro, T., 1999. A multiple-choice feeding-preference experiment utilising seagrasses with a natural population of herbivorous fishes. Mar. Ecol. Prog. Ser. 189, 295–299. https://doi.org/10.3354/meps189295.

Marone, J.L., Croone, E., 2006. Herbivory: effects on plant abundance, distribution and population growth. Proc. Roy. Soc. B. 273, 2575–2584. https://doi.org/10.1098/rspb.2006.3587.

Martínez-Crego, B., Arteaga, P., Tomas, F., Santos, R., 2016. The role of seagrass traits in mediating *Zostera noltei* vulnerability to mesograzers. PloS One 11, e0156848. https://doi.org/10.1371/journal.pone.0156848.

Martínez-Crego, B., Olivé, I., Santos, R., 2014. CO_2 and nutrient-driven changes across multiple levels of organization in *Zostera noltii* ecosystems. Biogeosciences 11, 7237–7249. https://doi.org/10.5194/bg-11-7237-2014.

Mazzella, L., Buia, M.C., Gambi, M.C., Lorenti, M., Russo, G.F., Scipi-one, M.B., Zupo, V., 1992. Plant–animal trophic relationships in the *Posidonia oceanica* ecosystem of the Mediterranean Sea: a review. In: John, D.M., Hawkins, S.J., Price, J.H. (Eds.), Plant–animal Interactions in the Marine Benthos, vol. 46. Clarendon Press, Oxford, pp. 165–187.

McClintock, J.B., 1986. On estimating energetic values of prey: implications in optimal diet models. Oecologia 70, 161–162. https://doi.org/10.1007/BF00377127.

McKone, K.L., Tanner, C.E., 2009. Role of salinity in the susceptibility of eelgrass *Zostera marina* to the wasting disease pathogen *Labyrinthula zosterae*. Mar. Ecol. Prog. Ser. 377, 123–130. https://doi.org/10.3354/meps07860.

Meyer, K.D., Paul, V.J., Sanger, H.R., Nelson, S.G., 1994. Effects of seaweed extracts and secondary metabolites on feeding by the herbivorous surgeonfish *Naso lituratus*. Coral Reefs 13, 105–112. https://doi.org/10.1007/BF00300770.

Moran, K.L., Bjorndal, K.A., 2007. Simulated green turtle grazing affects nutrient composition of the seagrass *Thalassia testudinum*. Mar. Biol. 150, 1083–1092. https://doi.org/10.1007/s00227-006-0427-9.

Papazian, S., Parrot, D., Burýšková, B., Weinberger, F., Tasdemir, D., 2019. Surface chemical defence of the eelgrass *Zostera marina* against microbial foulers. Sci. Rep. 9, 1–12. https://doi.org/10.1038/s41598-019-39212-3.

Paul, V.J., Cruz-Rivera, E., Thacker, R.W., 2001. Chemical mediation of macroalgal-herbivore interactions: ecological and evolutionary perspectives. In: McClintock, J., Baker, B. (Eds.), Mar. Chem. Ecol. CRC Press, Boca Raton, pp. 227–265.

Paul, V.J., Nelson, S.G., Sanger, H.R., 1990. Feeding preferences of adult and juvenile rabbitfish *Siganus argenteus* in relation to chemical defenses of tropical seaweeds. Mar. Ecol. Prog. Ser. 60, 23–34.

Pérez, M., Romero, J., 1994. Growth dynamics, production, and nutrient status of the seagrass *Cymodocea nodosa* in a Mediterranean semi-estuarine environment. Mar. Ecol. 15, 51–64. https://doi.org/10.1111/j.1439-0485.1994.tb00041.x.

Petersen, M., 2013. Rosmarinic acid: new aspects. Phytochemistry Rev. 12, 207–227.

Petersen, M., Abdullah, Y., Benner, J., Eberle, D., Gehlen, K., Hücherig, S., Janiak, V., Kim, K.H., Snader, M., Weitzel, C., Wolters, S., 2009. Evolution of rosmarinic acid biosynthesis. Phtochemistry 70, 1663–1679. https://doi.org/10.1016/j.phytochem.2009.05.010.

Peterson, C.H., Renaud, P.E., 1989. Analysis of feeding preference experiments. Oecologia 80, 82–86. https://doi.org/10.1007/BF00789935.

Pirc, H., 1989. Seasonal changes in soluble carbohydrates, starch, and energy content in Mediterranean seagrasses. Mar. Ecol. 10, 97–105. https://doi.org/10.1111/j.1439-0485.1989.tb00068.x.

Poore, A.G.B., Campbell, A.H., Coleman, R.A., Edgar, G.J., Jormalainen, V., Reynolds, P. L., Sotka, E.E., Stachowicz, J.J., Taylor, R.B., Vanderklift, M.A., Duffy, J.E., 2012. Global patterns in the impact of marine herbivores on benthic primary producers. Ecol. Lett. 15, 912–922. https://doi.org/10.1111/j.1461-0248.2012.01804.x.

Prado, P., Alcoverro, T., Romero, J., 2010. Influence of nutrients in the feeding ecology of seagrass (*Posidonia oceanica L.*) consumers: a stable isotopes approach. Mar. Biol. 157, 715–724. https://doi.org/10.1007/s00227-009-1355-2.

Prado, P., Heck Jr., K.L., 2011. Seagrass selection by omnivorous and herbivorous consumers: determining factors. Mar. Ecol. Prog. Ser. 429, 45–55. https://doi.org/10.3354/meps09076.

Preen, A., 1995. Impacts of dugong foraging on seagrass habitats: observational and experimental evidence for cultivation grazing. Mar. Ecol. Prog. Ser. 124, 201–213. https://doi.org/10.3354/meps124201.

Prince, J.S., LeBlanc, W.G., Macia, S., 2004. Design and analysis of multiple choice feeding data. Oecologia 138, 1–4. https://doi.org/10.1007/s00442-003-1413-2.

Roa, R., 1992. Design and analysis of multiple-choice feeding-preference experiments. Oecologia 89, 509–515. https://doi.org/10.1007/BF00317157.

Rodríguez, A., Clemente, S., Hernández, J.C., Brito, A., García, I., Becerro, M.A., 2017. Nutritional, structural and chemical defenses of common algae species against juvenile sea urchins. Mar. Biol. 164, 127. https://doi.org/10.1007/s00227-017-3156-3.

Salminen, J.P., Karonen, M., 2011. Chemical ecology of tannins and other phenolics: we need a change in approach. Funct. Ecol. 25, 325–338. https://doi.org/10.1111/j.1365-2435.2010.01826.x.

Schlosser, S.C., Lupatsch, I., Lawrence, J.M., Lawrence, A.L., Shpigel, M., 2005. Protein and energy digestibility and gonad development of the European sea urchin *Paracentrotus lividus* (Lamarck) fed algal and prepared diets during spring and fall. Aquacult. Res. 36, 972–982. https://doi.org/10.1111/j.1365-2109.2005.01306.x.

Short, F.T., Carruthers, T., Dennison, W., Waycott, M., 2007. Global seagrass distribution and diversity: a bioregional model. J. Exp. Mar. Biol. Ecol. 350, 3–20. https://doi.org/10.1016/j.jembe.2007.06.012.

Short, F.T., Polidoro, B., Livingstone, S.R., Carpenter, K.E., Bandeira, S., Bujang, J.S., Calumpong, H.P., Carruthers, T.J.B., Coles, R.G., Dennison, W.C., Erftemeijer, P.L.A., Fortes, M.D., Freeman, A.S., Jagtap, T.G., Kamal, A.H.M., Kendrick, G.A., Kenworthy, W.J., La Nafie, Y.A., Zieman, J.C., 2011. Extinction risk assessment of the world's seagrass species. Biol. Conserv. 144, 1961–1971. https://doi.org/10.1016/j.biocon.2011.04.010.

Sieg, R.D., Kubanek, J., 2013. Chemical ecology of marine angiosperms: opportunities at the interface of marine and terrestrial systems. J. Chem. Ecol. 39, 687–711. https://doi.org/10.1007/s10886-013-0297-9.

Silva, J., Barrote, I., Costa, M.M., Albano, S., Santos, R., 2013. Physiological responses of *Zostera marina* and *Cymodocea nodosa* to light-limitation stress. PloS One 8, e81058. https://doi.org/10.1371/journal.pone.0081058.

Simmonds, M.S., Stevenson, P.C., Hanson, F.E., 2019. Rosmarinic acid in *Canna generalis* activates the medial deterrent chemosensory neurone and deters feeding in the tobacco hornworm *Manduca sexta*. Physiol. Entomol. 44, 140–147. https://doi.org/10.1111/phen.12284.

Singer, M.C., 2000. Reducing ambiguity in describing plant-insect interactions: "preference"," acceptability" and" electivity. Ecol. Lett. 3, 159–162.

Stalikas, C.D., 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. J. Separ. Sci. 30, 3268–3295. https://doi.org/10.1002/jssc.200700261.

Steele, L., Valentine, J.F., 2012. Idiosyncratic responses of seagrass phenolic production following sea urchin grazing. Mar. Ecol. Prog. Ser. 466, 81–92. https://doi.org/10.3354/meps09921.

Subhashini, P., Dilipan, E., Thangaradjou, T., Papenbrock, J., 2013. Bioactive natural products from marine angiosperms: abundance and functions. Nat. Prod. Bioprospect. 3, 129–136. https://doi.org/10.1007/s13659-013-0043-6.

Tejada, S., Deudero, S., Box, A., Sureda, A., 2013. Physiological response of the sea urchin *Paracentrotus lividus* fed with the seagrass *Posidonia oceanica* and the alien algae *Caulerpa racemosa* and *Lophocladia lallemandii*. Mar. Environ. Res. 83, 48–53. https://doi.org/10.1016/j.marenvres.2012.10.008.

Teles, Y.C., Souza, M.S.R., Souza, M.D.F.V.D., 2018. Sulphated flavonoids: biosynthesis, structures, and biological activities. Molecules 23, 480. https://doi.org/10.3390/molecules23020480.

Thayer, G.W., Bjorndal, K.A., Ogden, J.C., Williams, S.L., Zieman, J.C., 1984. Role of larger herbivores in seagrass communities. Estuaries 7, 351–376. https://doi.org/10.2307/1351619.

Tomás-Barberán, F.A., Harborne, J.B., Self, R., 1987. Twelve 6-oxygenated flavone sulphates from *Lippia nodiflora andL. canescens*. Phytochemistry 26, 2281–2284. https://doi.org/10.1016/S0031-9422(00)84701-9.

Tomas, F., Abbott, J.M., Steinberg, C., Balk, M., Williams, S.L., Stachowicz, J.J., 2011. Plant genotype and nitrogen loading influence seagrass productivity, biochemistry, and plant–herbivore interactions. Ecology 92, 1807–1817. https://doi.org/10.1890/10-2095.1.

Toth, G.B., Pavia, H., 2007. Induced herbivore resistance in seaweeds: a meta-analysis. J. Ecol. 95, 425–434. https://doi.org/10.1111/j.1365-2745.2007.01224.x.

Vadas, R.L., 1977. Preferential feeding: an optimization strategy in sea urchins. Ecol. Monogr. 47, 337–371. https://doi.org/10.2307/1942173.

Valentine, J.F., Duffy, J.E., 2006. The central role of grazing in seagrass ecology. In: Larkum, A.W.D., Orth, R.J., Duarte, C.M. (Eds.), Seagrasses: Biology, Ecology and Conservation. Springer, the Netherlands, pp. 463–501.

Valentine, J.F., Heck Jr., K.L., 2001. The role of leaf nitrogen content in determining turtlegrass (*Thalassia testudinum*) grazing by a generalized herbivore in the northeastern Gulf of Mexico. J. Exp. Mar. Biol. Ecol. 258, 65–86. https://doi.org/10.1016/S0022-0981(00)00342-7.

Vergara, J.J., García-Sánchez, M.P., Olivé, I., García-Marín, P., Brun, F.G., Pérez-Lloréns, J.L., Hernández, I., 2012. Seasonal functioning and dynamics of *Caulerpa prolifera* meadows in shallow areas: an integrated approach in Cadiz Bay Natural Park. Estuar. Coast Shelf Sci. 112, 255–264. https://doi.org/10.1016/j.ecss.2012.07.031.

Vergeer, L.H.T., Aarts, T.L., De Groot, J.D., 1995. The 'wasting disease' and the effect of abiotic factors (light intensity, temperature, salinity) and infection with *Labyrinthula zosterae* on the phenolic content of *Zostera marina* shoots. Aquat. Bot. 52, 35–44. https://doi.org/10.1016/0304-3770(95)00480-N.

Vergés, A., Alcoverro, T., Romero, J., 2011. Plant defences and the role of epibiosis in mediating within-plant feeding choices of seagrass consumers. Oecologia 166, 381–390. https://doi.org/10.1007/s00442-010-1830-y.

Vergés, A., Becerro, M.A., Alcoverro, T., Romero, J., 2007a. Experimental evidence of chemical deterrence against multiple herbivores in the seagrass *Posidonia oceanica*. Mar. Ecol. Prog. Ser. 343, 107–114. https://doi.org/10.3354/meps06885.

Vergés, A., Becerro, M.A., Alcoverro, T., Romero, J., 2007b. Variation in multiple traits of vegetative and reproductive seagrass tissues influences plant–herbivore interactions. Oecologia 151, 675–686. https://doi.org/10.1007/s00442-006-0606-x.

Vergés, A., Doropoulos, C., Czarnik, R., McMahon, K., Llonch, N., Poore, A.G., 2018. Latitudinal variation in seagrass herbivory: Global patterns and explanatory mechanisms. Glob. Ecol. 27, 1068–1079. https://doi.org/10.1111/geb.12767.

Villazán, B., Salo, T., Brun, F.G., Vergara, J.J., Pedersen, M.F., 2015. High ammonium availability amplifies the adverse effect of low salinity on eelgrass *Zostera marina*. Mar. Ecol. Prog. Ser. 536, 149–162. https://doi.org/10.3354/meps11435.

Walker, T.S., Bais, H.P., Déziel, E., Schweizer, H.P., Rahme, L.G., Fall, R., Vivanco, J.M., 2004. *Pseudomonas aeruginosa*-plant root interactions. Phatogenicity, biofilm formation, and root exudation. Plant Physiol 134, 320–331. https://doi.org/10.1104/pp.103.027888.

Widmer, T.L., Laurent, N., 2006. Plant extracts containing caffeic acid and rosmarinic acid inhibit zoospore germination of *Phytophthora* spp. pathogenic to *Theobroma cacao*. Eur. J. Plant Pathol. 115, 377. https://doi.org/10.1007/s10658-006-9024-5.

Zidorn, C., 2016. Secondary metabolites of seagrasses (Alismatales and Potamogetonales; Alismatidae): chemical diversity, bioactivity, and ecological function. Phytochemistry 124, 5–28. https://doi.org/10.1016/j.phytochem.2016.02.004.

Zieman, J.C., Iverson, R.L., Ogden, J.C., 1984. Herbivory effects on *Thalassia testudinum* leaf growth and nitrogen content. Mar. Ecol. Prog. Ser. 15, 151–158.